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13. ABSTRACT *(Maximum 200 Words)*

Deregulated expression of cyclin E may play a role in tumorigenesis through the induction of genomic instability. We generated mice heterozygous for the tumor suppressor p53 or Rb and with an inducible transgene of either wildtype human cyclin E or a hyperstable allele (T380A) expressed in the mammary epithelia. While no mammary tumors were observed in any Rb heterozygote strain, there was synergy between the hyperstable T380A transgene and p53 heterozygosity; increasing mammary tumor penetrance from 8% and 12% respectively in p53 and T380A controls to 50% in the doubly engineered mice ($p<0.0001$). However, despite the increase in tumor penetrence there was no decrease in latency with the onset of mammary tumorigenesis approximately corresponding to reproductive senescence. Tumor cells demonstrated loss of p53 heterozygosity and constitutive expression of the cyclin E transgene. These preliminary data strongly support the hypothesis that deregulated expression of cyclin E induces tumorigenesis through genomic instability and suggests that inactivation of p53 and deregulated expression of cyclin E may confer a growth advantage in mammary epithelial cells. However, in this mouse model, not unlike human sporadic cases, these tumors may not form until the onset of reproductive senescence when hormonal influence may play a key role.

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Introduction

The purpose of this project is to test whether deregulated cyclin E in the mammary epithelia predisposes to chromosomal instability and hence tumorigenesis. To test this hypothesis based on in vitro work (Spruck et al. 1999) we crossed transgenic mice expressing either wild-type human cyclin E or a hyperstable mutant (T380A) with mice heterozygous at either the p53 or Rb loci. If genomic instability is induced by deregulated expression in the mammary epithelia, we anticipate an increased penetrance and decreased latency of tumorigenesis. Neither heterozygosity of p53 or Rb predisposes to mammary tumorigenesis in mice (Jacks et al. 1992; Jacks et al. 1994; Harvey et al. 1995) except in the BALB/c background (Kuperwasser et al. 2000). In the transgenic mice 10% develop mammary tumors in the wildtype cyclin E strain (CycE) (Bortner and Rosenberg 1997) and 25% develop tumors in the hyperstable transgene strain ($T_{380}A$; unpublished). In both cases tumor appearance occurs no earlier than 11 months of age.

Body

In the second year of this project it was intended to meet several technical objectives which are ongoing and will reach completion in the third year (in boldface):

Technical objective 1.	Generation of founder mice
Task 1. Months 1-2.	Cross breed transgenic and heterozygous strains.
Task 2. Months 2-6.	Genotype offspring and identify mice appropriate as founders.
Technical objective 2.	Characterization of tumor kinetics and types
Task 3. Months 5-36.	**Weekly examination by palpitation and recording approximate tumor size.**
Task 4. Months 8-36.	**Terminal necropsy, tumor collection and histopathology.**
Technical objective 3.	Genomic instability
Task 5. Months 1-6.	Establishment of primary cell culture and chromosome **painting techniques.**
Task 6. Months 12-36	**Karyotype analysis of cells derived from 'normal' and tumor mammary specimens from all genotypes.**

All technical objectives set for the first and second 12 month periods have been achieved.

Characterization of tumor kinetics and types

During the last 12 month period (May 01 to May 02) the mammary and non-mammary tumor incidence has been studied in >300 mice with representation in all 9 of the genotypes to be studied. Data collected includes clinical observations made during routine animal surveillance and at necropsy with subsequent histopathology. The main focus was on mammary tumors but all pathology was recorded with greater attention paid to neoplastic lesions. A summary of the findings is presented in Table 1.

A number of important observations have been made during this period of the project but final conclusions cannot be reasonably drawn until completion of the study. However, a brief synopsis of each finding (as of May 02) is presented below. Also attached is a manuscript describing an interesting but incidental observation in the $Rb^{+/-}$ mice also transgenic for T380A.

The very nature of this project precludes definitive conclusions drawn at this stage. However, few technical issues have presented any significant problems; data acquisition and analysis should easily be achieved in the allotted time frame. All goals have been met and preliminary statistical analysis indicates a tentatively positive result.

Table 1. tumor incidence.

Genotype / Neoplasm	wt	$Rb^{+/-}$	$Rb^{+/-}$ CycE	$Rb^{+/-}$ T380A	$p53^{+/-}$	$p53^{+/-}$ CycE	$p53^{+/-}$ T380A	CycE	T380A
Mammary carcinoma*	-	-	-	-	8%	20%	50%	-	12%
Lymphoma[1]	4%	-	-	-	14%	20%	46%	2%	5%
Osteosarcoma	-	-	-	-	37%	45%	12%	-	-
Leiomyosarcoma	2%	-	-	-	4%	10%	4%	4%	2%
Poorly/Undifferentiated sarcoma	-	-	-	-	2%	-	12%	-	-
Pancreatic acinar cell carcinoma	-	-	-	-	2%	-	-	-	-
Rhabdomyosarcoma	-	-	-	-	2%	10%	4%	-	-
Haemangiosarcoma	-	-	-	2%	2%	-	-	-	-
Keratoacanthoma[2]	-	-	-	-	2%	-	-	-	-
Pituitary adenoma/carcinoma[3]	-	96%	86%	82%	8%	-	4%	-	-
Thyroid carcinoma[4]	2%	84%	71%	92%	-	-	-	-	-
Number of mice	57	50	28	50	49	20	26	50	42

*Includes carcinoma in situ. Mice bearing more than 1 mammary lesion count as a single data point. [1]Includes thymic, splenic and generalized lymphomas. [2]Keratoacanthoma of the pinna removed surgically, the mouse did not later develop further tumors. [3]Neoplasia of the pars intermedia in $Rb^{+/-}$ mice and pars distilis in the $p53^{+/-}$ mice. [4]Medullary thyroid carcinoma in Rb mice and follicular cell adenoma in a wild type mouse.

Cyclin E and T380A immunoreactivity correlates with nuclear atypia and hyperplasia in midlactation mammary glands

It has previously been shown that the cyclin E transgene in these mice is associated with hyperplasia in the mammary gland epithelia concomitant with its expression under the prolactin-responsive β-lactoglobulin promoter (Bortner and Rosenberg 1997). This phenomenon is transient, being associated with the proliferation of the epithelia during the lactogenic cycle, initially manifest in the third trimester and persisting until the litter is weaned. At this point the mammary epithelia undergo involution and the hyperplastic epithelia are largely included in this process. To further examine this phenotype and to characterize the phenotype of the hyperstable T380A transgenic mice we studied the mammary epithelia of midlactation (2 weeks post-partum) by standard histological haemotoxylin and eosin (H&E) staining and by immunohistochemistry using the human cyclin E-specific antibody HE12. By H&E staining hyperplasia appeared more widespread in T380A transgenic mice than those with the normal human cyclin E transgene but no more severe. When examined by HE12 immunohistochemistry, virtually all the mammary epithelia showed transgene expression but the more intense staining cells also appeared to be those either in hyperplastic projections and/or with enlarged nuclei, often showing signs of atypia such as envelope folds and pinches

Mammary tumors in transgenic mice or mice heterozygous at the Rb and p53 loci

Hyperplasia in the mammary epithelium is generally considered a preneoplastic lesion. In our transgenic strains without heterozygosity at either the Rb or p53 locus only a small percentage, 12%, proceeded to develop mammary tumors. Surprisingly, none of the CycE mice in the present study developed overt neoplastic lesions whereas in a C3H x C57BL/6J background we had observed approximately 10% (Bortner and Rosenberg 1997). This result is likely due to the greater representation of the C57BL/6J genetic background in the mice of the present study, which are generally less susceptible to mammary tumors than C3H mice. However, 6 T380A mice (12%) developed mammary adenocarcinoma by 18 months. The earliest age at which a T380A mouse presented with a mammary carcinoma was 11 months. Mammary tumors were not observed in $Rb^{+/-}$ mice before 1 year at which time pituitary pars intermedia or medullary thyroid lesions usually were contributing profoundly to morbidity or mortality rates. Four $p53^{+/-}$ mice (8%) developed mammary adenocarcinoma before 18 months with the earliest manifestation observed at 13 months. Histologically, most of the mammary tumors were composed of dense sheets or nests of neoplastic cells occasionally interspersed with fine trabeculae. In two $p53^{+/-}$ mice the mammary lesions showed areas of squamous metaplasia and keratin deposition characteristic of adenoacanthoma which was not observed in any other genotype. Nuclear/cytoplasmic ratios, nuclear atypia,

necrosis and mitotic rates were moderately high. Local invasion of the overlying integument or underlying musculature were a frequent observation. Lymphatic dissemination was infrequent and metastases to the lung, liver or marrow were not observed. Carcinomas in situ were not observed and only one individual $p53^{+/-}$ animal presented with more than one neoplastic lesion.

The tumor spectrum in $Rb^{+/-}$ or $p53^{+/-}$ mice is unaffected by cyclin E transgenes

The spectrum of tumor susceptibility in $Rb^{+/-}$ and $p53^{+/-}$ mice is well established although we are not aware of a systematic study of female mice for either strain. In both strains, we observed a tumor spectrum similar to those of published reports (Lee et al. 1992; Jacks et al. 1994; Harrison et al. 1995; Harvey et al. 1995; Park et al. 1999). The predominant lesions in $Rb^{+/-}$ mice were pituitary tumors and medullary thyroid carcinomas with frequencies comparable to those in the literature. Likewise, $p53^{+/-}$ mice exhibited a similar sarcoma tumor spectrum to that published with osteosarcomas, lymphomas and rhabdomyosarcomas frequently represented. In addition we observed a moderate rate of mammary carcinoma (8% n=4) and pituitary pars distilis carcinoma (8% n=4), the latter of which were always accompanied by galactorrhea. When either the hyperstable or wild type allele of cyclin E was also present the tumor suppressor spectrum of tumors was similar to those without the transgene. This is indicative that there was no significant alteration of tumor pattern although the frequency of mammary tumors and possibly of lymphomas was enhanced in the mice bearing the hyperstable T380A allele (see below).

$Rb^{+/-}$ mice do not succumb to mammary tumors

Mammary tumors were not observed in any strain of mice bearing an Rb mutation. Pituitary adenomas were a frequent lesion encountered in all three $Rb^{+/-}$ strains and were frequently the cause for premature euthanasia. Although the lesions were always histologically consistent with cells of a pars intermedia origin, several mice exhibited excessive mammary development at necropsy, even to the point of overt galactorrhea in some cases, suggestive of prolactinaemia. Prolactin immunohistochemistry proved largely negative in these lesions although a few lesions demonstrated a weak diffuse positive staining in some tumor cells. A common theme to the cases of galatorrhea were a seemingly functional pars distilis, often completely ablated in the larger tumors, with an intermediate sized pars intermedia lesion (3-4mm in the longest dimension). We interpret these cases as a destruction of the hypophyseal stalk and consequently the normal regulatory suppression of prolactin secretion by prolactin inhibitory factor from the hypothalamus is lifted. Nevertheless, even in these cases of overt prolactinaemia, mammary tumors were not observed with or without the presence of a prolactin sensitive cyclin E transgene. The age of onset, frequency, histological grade and morphology were comparable between each of the three $Rb^{+/-}$ bearing mouse strains. Similarly, medullary thyroid carcinomas were observed in approximately 75% of mice with Rb mutations. Clinical morbidity due to thyroid tumors was an infrequent complication. However in some cases mice exhibited involuntary vocalization due to compression of the trachea. Immunohistochemistry demonstrated consistently positive calcitonin immunoreactivity and negative transgene immunoreactivity. A manuscript for an interesting incidental finding of hyperpigmentation in some of these mice is attached.

p53 heterozygosity acts synergistically with T380A to promote mammary tumorigenesis and decreases life expectancy

Even though the tumor spectrum was unaltered in mice bearing the $p53^{+/-}$ mutation with or without cyclin E transgenes, the frequency with which they presented was markedly changed in the presence of the hyperstable cyclin E transgene. The frequency of mammary tumorigenesis in $p53^{+/-}$ mice without a transgene was 8% (4/50). In mice with the wildtype cyclin E transgene this was fractionally increased. However, in mice bearing the hyperstable allele of cyclin E the penetrence was increased to 50%. This effect was significantly greater than any additive effect as the mammary tumor frequency in the transgenic mice for the hyperstable allele in the absence of a p53 mutation was merely 12% (χ^2 =15.8; p<0.0001). However, the age of onset was not significantly changed. All mammary carcinomas were high grade with high mitotic rates, considerable nuclear atypia and areas of necrosis. Local invasion of the skin and/or musculature were usually observed. However, despite the poor differentiation and obvious malignant biological behavior, hematogenous metastases were not observed, despite routine histological examination of lung, liver and marrow. Lymphatic dissemination was observed in several cases with large mammary lesions but was a generally infrequent observation.

Mammary tumors in $p53^{+/-}$T380A mice tended to present as aggressive fast growing lesions. Typically the time from first presentation to a size of 1cm was 2-6 days whereas lesions in T380A or $p53^{+/-}$ mice took 3-8 days. Furthermore, several of $p53^{+/-}$T380A lesions exhibited extraordinarily high mitotic indices, >30 mitotic figures per 40x objective field, and large areas of necrosis. By contrast lesions of T380A or $p53^{+/-}$ mice were typified by more moderate mitotic rates of 4-15 figures per 40x objective field. Several mice were found to have multiple neoplastic lesions including 3 mice with either 2 independent mammary carcinomas or 1 mammary carcinoma and a carcinoma in situ. Indeed, one of these mice presented with 4 independent neoplastic lesions; 2 mammary carcinomas in an inguinal and contralateral abdominal gland, splenic lymphoma and a large poorly differentiated thoracic sarcoma. In F1 generation $p53^{+/-}$T380A mice, where each mouse is heterozygous at the locus of transgene insertion, mammary tumourigenesis was observed at an equivalent rate; 60% (3/5) indicating that the level of expression may not be the decisive factor, rather the expression throughout the cell cycle.

Survival curves of $p53^{+/-}$T380A mice showed a reduced life expectancy over both $p53^{+/-}$ and T380A mice. Whereas approximately 30% of $p53^{+/-}$ and 60% of T380A mice were tumor-free at 1.5 years, no $p53^{+/-}$ T380A animal has reached this age tumor-free. Although some reduction in life expectancy was anticipated due to the increased numbers of mice succumbing to mammary tumors after 11 months, there also appeared to be an earlier onset for lymphomas, particularly thymic lymphomas, and osteosarcomas, particularly of the vertebrae. The incidence and latency of susceptibility to other $p53^{+/-}$ spectrum tumors did not appear different.

Mammary tumors show loss of p53, anueploidy and constitutive expression of the cyclin E transgene

The preliminary analysis for loss of p53 heterozygosity was determined in tumors by PCR. Expression of the cyclin E transgene in tumors was determined by immunohistochemistry. In all mammary tumors tested by PCR to date p53 appeared to be lost, including those of the T380A mice which carried 2 copies of the wildtype allele. Loss of Rb was infrequent. Furthermore, all mammary tumours tested to date show constitutive expression of the transgene (Fig 1). Karyotypic analysis of all tumor cell lines established to data demonstrates considerable aneuploidy with chromosome counts of 62-97 (normal count 2n=40). Cells in culture also exhibit varying degrees of karyomegaly, are frequently multinuecleate (in one line up to 7 nuclei/cell) and are unstable in culture. Further analysis by southern analysis and chromosome painting is ongoing.

Cyclin E transgenes are not expressed in other tumor types

Representative sections from non-mammary tumors were tested by immunohistochemistry for transgene expression. In all cases tested to date HE12 immunoreactivity was negative whilst 10-20% of the tumor cell population tested positive for endogenous cyclin E with the M20 antibody (Fig.2).

Summary

Preliminary statistical analysis demonstrates clear synergy between p53 heterozygosity and transgenic expression of the hyperstable form of cyclin E (T380A). While there appears that there is also synergy with the normal form of cyclin E this has not reached statistical significance with only approximately 40% of these mice studied. The increased penetrence of mammary lesions in bitransgenic mice and apparent loss of tumor suppressor loci is indicative that the mechanism of cyclin E induced tumourigenesis is indeed through genomic instability. The latency does not appear to be dramatically reduced but no conclusions can be drawn until the final analysis. We are currently exploring the hypothesis that early genetic lesions take place during transgene expression in the final trimester of pregnancy and during lactation but are largely ablated by mammary epithelial involution. Persistence of hyperplastic alveolar nodules beyond the period of transgene expression probably represent preneoplastic lesions which are then induced to malignancy during the early period of reproductive senescence at which time transgene stimulation again occurs with hormone (prolactin) secretion.

Key Research Accomplishments

Established in months 13-24;

- Animal breeding complete
- Substantial proportion of mice taken to the final time point
- Immunohistochemistry established for transgenes and other relevant antigens.
- Karyotype analysis initiated and underway.

Reportable Outcomes

- Establishment of a new model of murine mammary tumorigenesis
- Preliminary demonstration of synergy between p53 heterozygosity and transgenic expression of the hyperstable form of cyclin E
- An incidental finding of hyperpigmentation in $Rb^{+/-}$ mice with pituitary pars intermedia lesions.

Conclusions

The initial objectives of this project have been met with few technical problems. Characterization of all tumors encountered to date has been relatively straightforward. More involved histopathological diagnoses have been presented at comparative pathology rounds held at TSRI in collaboration with Dr Kent Osborn (veterinary pathologist). Greater than 70% of the mice needed for this study have reached the study endpoint. Ancillary diagnostics are in place and no insurmountable technical difficulties have arisen. Completion of the necropsies is anticipated within a few months. Completion of karyotype analysis and correlations between clinical, histologic and biochemical determinations will be made upon completion of data acquisition but are expected to be complete comfortably before May 2003, the due date for completion of these studies.

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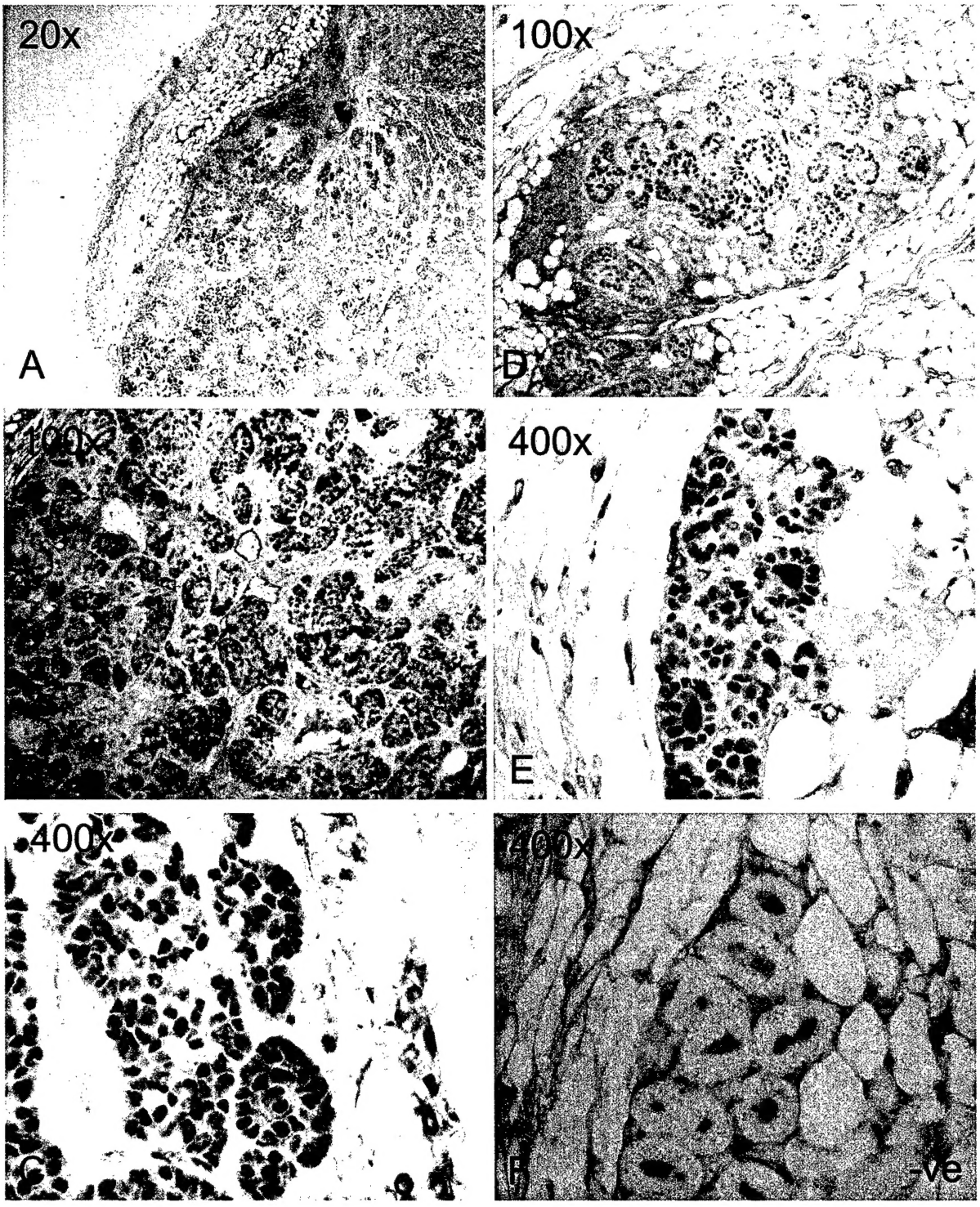

Figure 1. Transgene immunohistochemistry in mammary lesions. Representative sections from mammary carcinoma (A-C) and hyperplastic alveolar nodules in aged mice (D and E) were incubated with HE12 mouse monoclonal anti-human cyclin E IgG. In the negative control (F) the secondary rabbit anti-mouse IgG reacts with endogenous immunoglobulins but not the epithelial nuclei. Transgene cyclin E expression is confined to the nucleus and is masked by nuclear counterstains hence the absence of haematoxylin.

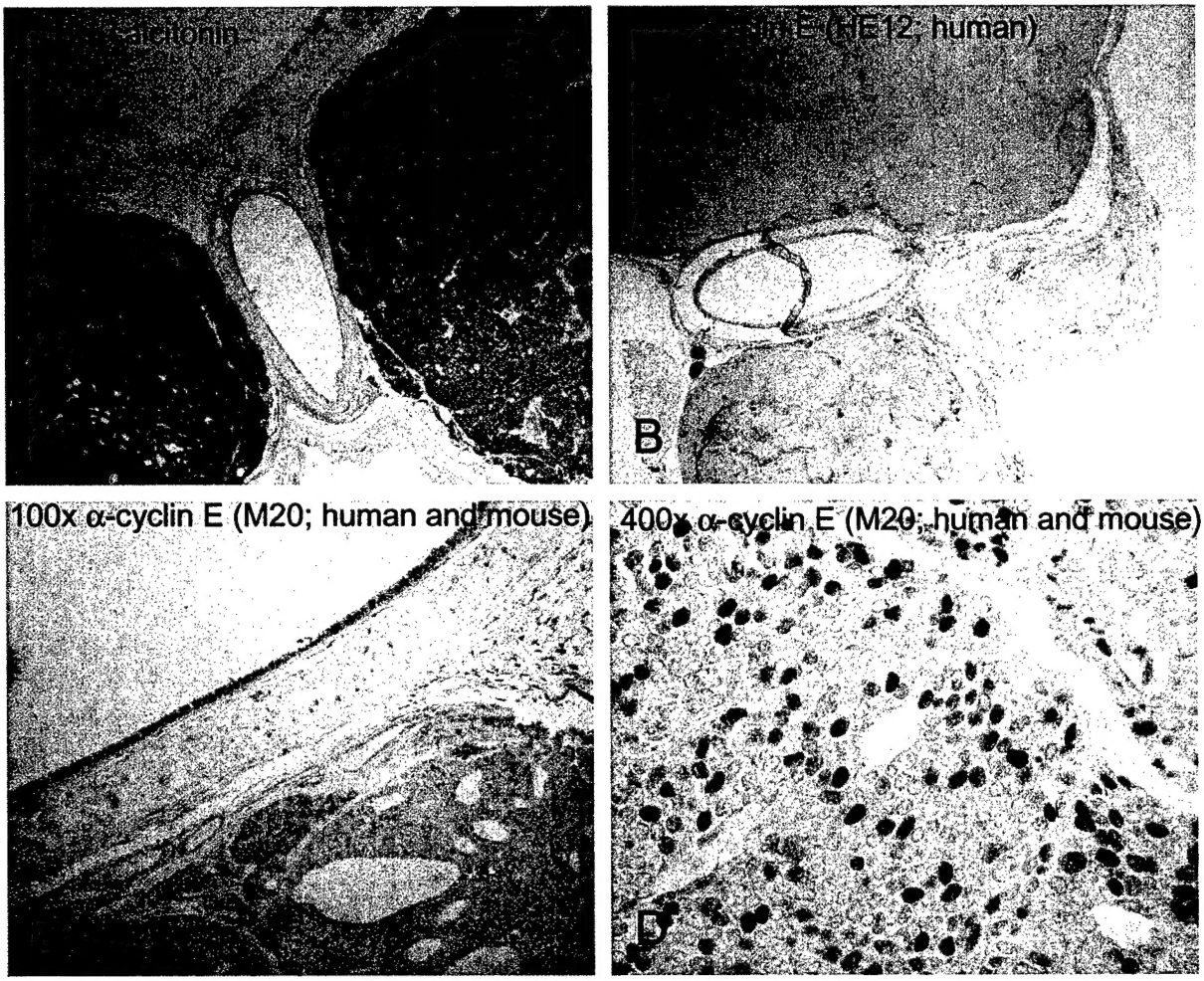

Figure 2. The cyclin E transgene is not expressed in non-mammary tumours. Sections from an $Rb^{+/-}$ medullary thyroid carcinoma. A) probed with rabbit anticalcitonin IgG showing strong immunoreactivity in the neoplastic cells but none in the surrounding normal tissue. B) negative staining for human cyclin E with the HE12 mouse monoclonal anti human cyclin E IgG. C and D. Positive staining for M20 immunoreactivity in approximately 20% of the neoplastic cells. M20 is a rabbit polyclonal IgG that reacts with both human and mouse cyclin E.

Hyperpigmentation associated with pituitary pars intermedia proliferative lesions revealed in yellow pelage $Rb^{+/-}$ and $p27^{-/-}$ female mice.

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Key words: pituitary adenoma, pars intermedia, hyperpigmentation, melanocyte stimulating hormone, retinoblastoma, $p27^{Kip1}$

Submitted to *Comparative Medicine* as a case report.

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Abstract

Spontaneous proliferative lesions of the pituitary pars intermedia are infrequent in wild-type mice. However, these lesions occur frequently in 2 strains of genetically engineered mice. Mice nullizygous at the retinoblastoma tumor suppressor locus die *in utero* while mice heterozygous at the Rb-1 locus develop normally but succumb to adenomas of the pituitary pars intermedia and medullary thyroid carcinoma by approximately 1 year of age. Similarly mice nullizygous for p27 develop pars intermedia hyperplasia by approximately 6 months. In a study outcrossing these mice from BL6 (black coat color) and BL6x129 (agouti) backgrounds, respectively, to one with yellow pelage, pituitary tumor formation was associated with the appearance of skin and fur hyperpigmentation. This phenomenon was first manifest in the vibrissae and subsequently, 1-2 weeks, later as periorbital and dorsal patches and was associated with pituitary lesions greater than 4 mm in size. In $Rb^{+/-}$ mice hyperpigmentation preceded morbidity from the tumors by 2-4 weeks while in $p27^{-/-}$ mice the onset of hyperpigmentation was earlier, more gradual and prolonged. Changes in the adrenal zona fasciculata parallel Addison's and Nelson's syndromes in man. Hyperpigmentation provides a means by which these proliferative lesions of the pars intermedia can be monitored in live mice which is not possible in albino strains or those with black or agouti pelage.

Introduction

Spontaneous pituitary tumors in mice are a fairly frequent occurrence but usually present in the anterior pituitary (pars distilis) and often present with hormonally distinct systemic phenotypes such as galactorrhea with prolactinoma. Spontaneous lesions of the pars intermedia are a rare occurance in non-genetically engineered mice but occur with almost 100% penetrance in mice heterozygous at the RB-1 tumor suppressor locus (1) or nullizygous at the $p27^{Kip1}$ cyclin-dependent kinase inhibitor locus (2-5).

Pituitary adenomas in $Rb^{+/-}$ mice are associated with loss of the remaining Rb wild type allele in cells at an early age and subsequent proliferative lesions appear to produce melanocyte stimulating hormone (MSH). Similarly, pars intermedia adenomas in p27 nullizygous mice appear early, usually detectable histologically in mice under 8 weeks of age. However, most genetically engineered mice are produced in strains of mice with dark fur; either black (BL6) or agouti (129 etc) and the effects of possible MSH overproduction in the skin are masked.

In this brief case report we present evidence that adenomas of the pars intermedia in both $Rb^{+/-}$ and $p27^{-/-}$ mice are associated with a hyperpigmentation phenotype only revealed when these genetic alterations are introduced into a mouse strain with yellow pelage.

Case Reports

As part of a mammary tumorigenesis study, $Rb^{+/-}$ mice in a C57/BL6 background (1) and $p27^{-/-}$ mice in a 129SV background (5) (black and agouti fur coloration respectively), were crossed to a transgenic strain in a C57/BL6 x C3H background with yellow pelage. The yellow pelage mice were created by integration of a transgene for the agouti gene product under the K14 promoter the expression of which is mainly restricted to integumentary keratinocytes (6). These mice were also transgenic for a human cyclin E allele under the BLG promoter, the expression of which is confined to the lactiferous mammary epithelia during the third trimester of pregnancy and lactation but does not influence the development of pituitary neoplasia. The pelage of these mice is a solid and uniform yellow with only slightly lighter coloration ventrally. The vibrissae are yellow and the eyes are black (6).

As the study was primarily of mammary tumorigenesis only female mice were studied. After 2 pregnancies (used to activate the cyclin E transgene in the mammary epithelia) each $Rb^{+/-}$ mouse was aged to 1 year. $p27^{-/-}$ mice are not able to become pregnant but were maintained in a breeder cage for 2 months and then aged in isolation. The mice were inspected twice weekly for signs of mammary tumorigenesis or any other notable clinical symptoms. At 1 year, or sooner if quality of life was deemed poor, the mice were euthanized by CO_2 asphyxia subsequent to O_2/CO_2 (1:1) anesthesia.

Each mouse underwent a full necropsy which included, but was not limited to, routine histological examination of the mammary glands and pituitary gland. Tissues were fixed in 10% neutral buffered formalin for 24hr, embedded in paraffin, sectioned to 8μm and stained with hematoxylin and eosin by standard methods. The left adrenal glands for each mouse was dissected free from the kidney and perirenal adipose, and weighed post-fixation. A total of 52 $Rb^{+/-}$ and 4 $p27^{-/-}$ mice were studied.

All of the animals in this study were maintained at 1-2 animals/cage in a 12:12 light dark cycle with *ad libitum* access to food and water in compliance with, and approved by, The Scripps Research Institute (TSRI) Animal Research Committee. TSRI is a fully accredited AAALAC institution.

Results

At 1 year of age, 88% of $Rb^{+/-}$ mice had pituitary proliferative lesions as determined by histological examination of H&E stained sections (46/52). Of these, 2 were malignant and showed unequivocal invasion of the brain. Of the remaining 44, 34 were benign and were classified as adenomas, while 10 showed aggressive cytological features such as high mitotic rate, nuclear atypia and necrosis, though invasion of the overlying brain was not present (Figure 1).

30% of these mice (n=14) exhibited hyperpigmentation (see below and Figure 2). 7% (n=3) exhibited galactorrhea and diffuse mammary alveolar hyperplasia without hyperpigmentation, although the pituitary tumors morphologically resembled adenoma consistent with pars intermedia origin. 4% (n=2) had small tumors but were euthanised because of morbity associated with hydrocephalus (no hyperpigmentation). Physical measurements of the tumors (longest dimension) were greater in mice with hyperpigmentation 5.3±1.9 (n=14) *vs.* 2.4±1.3 mm (n=33) (mean±SD; $p<0.001$). Tumors with a longest dimension measurement of >4mm were always associated with hyperpigmentation with the exception of one. Histologically, the single tumor of 5 mm which was not associated with hyperpigmentation, or galactorrhea, was largely haemorrhagic with little volume of the lesion actually composed of neoplastic cells. Hyperpigmentation was also associated with higher grade lesions as both malignant tumors and 7/10 of the "aggressive" adenomas were associated with hyperpigmentation.

Left adrenal gland mass did not correlate with the size of the pituitary lesion ($r=0.09$; n=47) or differ between animals with (3.75 ± 0.69 mg) or without (3.90 ± 0.71 mg) hyperpigmentation ($p>0.5$; n=13 and 33 respectively). However, there appeared to be a hypertrophic change in the zona fasciculata of each mouse with hyperpigmentation and the cytoplasm appeared more solidly eosinophilic than those in mice without hyperpigmentation (Figure 3). Mice with hyperpigmentation always contained a vast majority of these cells lacking the finely vacuolated cytoplasm of the normal adrenal zona fasciculata.

The onset of hyperpigmentation in $Rb^{+/-}$ mice was usually manifest initially in darkening of the vibrissae followed 2-14 days later with periorbital and/or dorsolateral patches with few ventral patches. Rarely did greater than 20% of the coat turn dark before the development of morbidity, often within 4 weeks of the first instance of hyperpigmentation. Terminal symptoms included doming of the skull (compression of the third ventricle with hydrocephalus), emaciation and poor conditioning with a moribund attitude.

In 4 $p27^{-/-}$ mice studied, hyperpigmentation appeared at 6 months and steadily increased with age. In these mice more than 80% of the dorsal pelage usually changed color before clinical morbidity was observed. Histopathological examination of the pituitary lesions in these mice revealed hyperplastic/benign lesions with a generally lower mitotic rate and few instances the more aggressive cytological markers of nuclear atypia and/or necrosis. The adrenals of $p27^{-/-}$ mice were approximately 2-3 fold more massive than the $Rb^{+/-}$ adrenals which was easily accounted for by medullary hyperplasia, part of the multiorgan hyperplasia associated with this genotype. However, the zona fasciculata of hyperpigmented $p27^{-/-}$ mice appeared to be composed of a mixture of both cells with a solidly eosinophilic cytoplasm and the normal finely vacuolated cytoplasm and in some instances cells with a cytoplasm displaying areas of both kinds.

Discussion

We report here a phenotype of hyperpigmentation in mice with melanotroph hyperplasias/adenomas revealed in mice with yellow pelage. The phenotypic characterization of $Rb^{+/-}$ and $p27^{-/-}$ genotypes in dark pelage mice have been fully described elsewhere (1-5). Hyperpigmentation in these mice probably occurs as a result of competition between the localized expression of the agouti gene product and circulating MSH synthesized in the pars intermedia melanotrophs. Hyperplasia or adenoma of the pars intermedia increases the melanotroph population and therefore elevates circulating MSH. The slower and earlier onset but more profound hyperpigmentation in $p27^{-/-}$ mice probably reflects the fact that while these mice all develop pituitary hyperplasia only approximately 50% develop overt pars intermedia neoplasia (2). This is supported by the observation of hyperpigmentation in all the $p27^{-/-}$ mice studied but the long latency of morbidity in the absence of overt neoplasia in any of the 4 mice. Conversely, the frequency of adenoma formation in $Rb^{+/-}$ mice is almost 100% mice with a lifespan restricted only by genotypic limits. Loss of the remaining wild type Rb allele occurs early but subsequent genetic lesions are a largely stochastic process and latency is long and variable (1). Indeed many of the $Rb^{+/-}$ mice were euthanised at ages <10 months due to complications associated with medullary thyroid carcinomas without either overt pituitary hyperplasia or hyperpigmentation. In 3 $Rb^{+/-}$ mice, large pituitary adenomas were associated with galactorrhea in the absence of hyperpigmentation despite histological morphology ('foamy' lightly eosinophylic cytoplasm) consistent with a pars intermedia origin. In the absence of ancillary diagnostics we tentatively interpret these cases as hormonally inactive pars intermedia adenomas with physical destruction of the bridge with the hypothalamus known to secrete prolactin inhibitory factor. In each of these 3 cases, histological examination revealed the presence of some normal pars distilis in which the mammotrophs are located.

Yellow pelage in mice is usually associated with a dominant mutation of the agouti gene promoter resulting in constitutive expression of the agouti gene product (7). Constitutive expression disrupts the normal alternating banding of eumelanin (black) and pheomelanin (yellow) in the hair fibers creating the wild-type agouti pelage (reviewed by Wolff ref. (8). In addition, at least with the two dominant alleles best characterized, these mutations also result in ectopic expression of the agouti gene product in numerous tissues other than skin. As the agouti gene product is a potent inhibitor of proopiomelanocortin (POMC) signaling in the brain (9), ectopic expression results in 'yellow obese mouse syndrome' with the associated phenotypes of hyperphagia, hyperinsulinemia, hyperglycemia and increased susceptibility to mammary, urinary bladder, liver and lung tumorigenesis (8). In the mice of this study, however, the expression of the agouti gene product is largely restricted to the keratinocytes in the skin (6) and therefore these mice do not show a phenotype of type II diabetes or tumorigenic susceptibility other than those associated with the $Rb^{+/-}$ and $p27^{-/-}$ genotype.

While we have not directly compared melanin deposition with wild-type black mice, we have used the term hyperpigmentation to describe this phenomenon because of parallels with the human condition. In man, hyperpigmentation results from i) primary adrenal insuficiency (Addison's disease) or ii) secondary to adrenalectomy as a necessary consequence of cortisol hypersecretion (Nelson's syndrome). The adrenal histology, at least in the $Rb^{+/-}$ mice, is consistent with adrenal dysfunction secondary to the formation of pituitary lesions. Whether the normal endocrine feedback loop is broken because the ACTH/MSH overproduction outstrips the adrenal cortosol production or the adrenals fail is unclear but the relatively acute onset of hyperpigmentation and zona fasciculata cytology might suggest that the latter is more likely. Such endocrinology is beyond the scope of these incidental findings but might be easily determined by immunoassay of circulating ACTH and cortisol.

The rostral to caudal pattern of hyperpigmentation may be influenced by the grooming pattern in mice. Typically the vibrissae and periorbital regions are frequently groomed while more caudal areas are groomed less. Shaving of K14iA mice does not result in eumelanin pigmentation while plucking does. In the few instances where these mice have developed dorsocervical dermatitis the areas surrounding the lesions appeared to be eumelanic. Clearly the pigmentation phenotype can be influenced by both local and circulating factors.

Mice transgenic for the agouti gene under the K14 promoter (K14iA) were originally developed to generate a visual means of following transgene inheritance; constructs co-transfected with K14iA integrate into the same locus in tandem (6). As the expression of the agouti product is confined to the skin of K14iA mice, these mice have the yellow pelage in the absence of the other phenotypes associated with dominant mutants of the agouti promoter. Therefore, monitoring the level of hyperpigmentation in these mice provides a means of visually assessing pituitary lesion formation in otherwise healthy live mice which is not possible in albino strains or in strains with black or agouti pelage.

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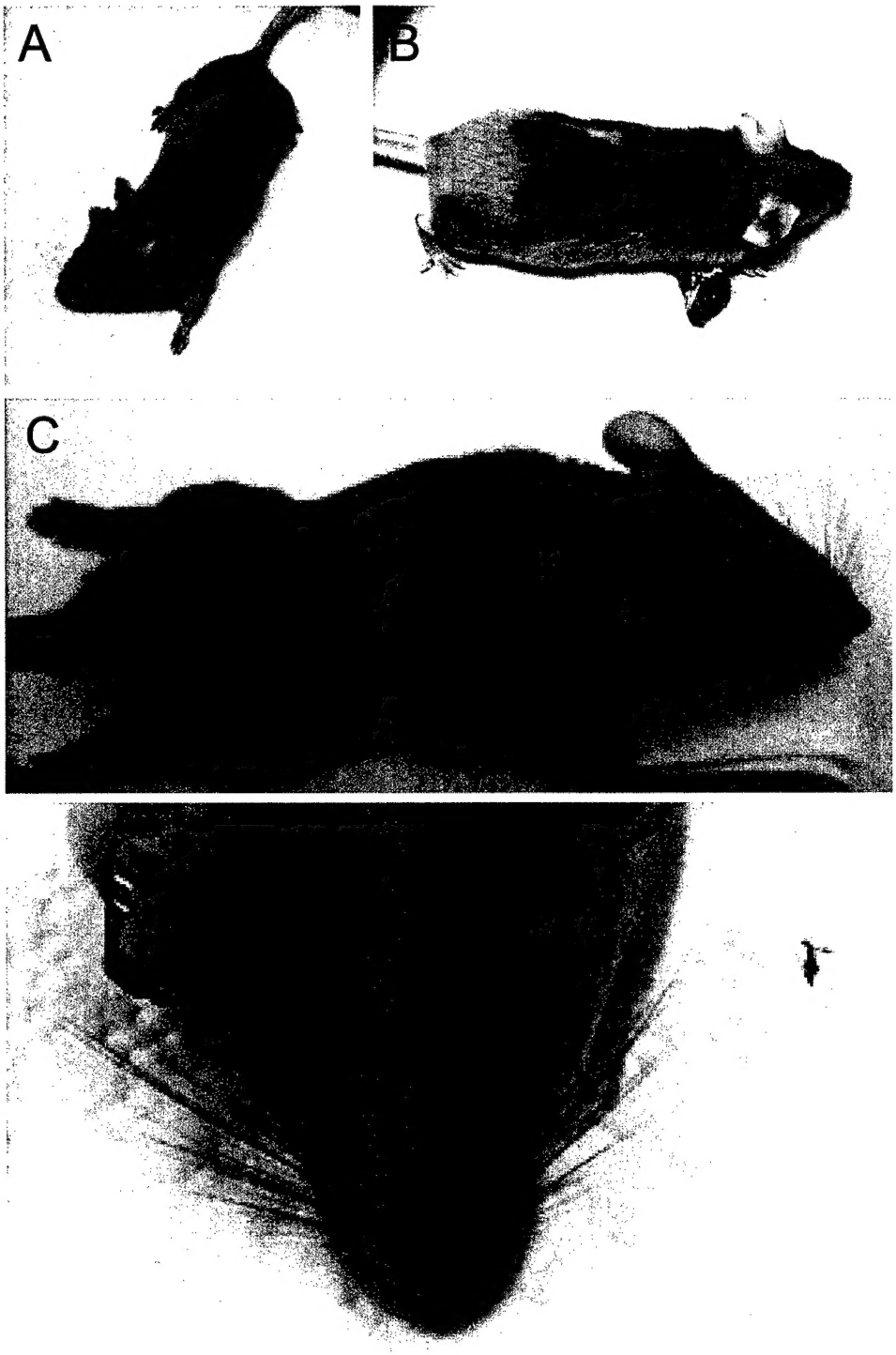
Figure legends

Figure 1. Pituitary tumours in Rb+/- mice at 20x and 200x magnification. A and B. Carcinoma exhibiting invasion of the overlying brain, nuclear atypia, areas of necrosis and a high mitotic rate. C and D. Aggressive adenoma with no invasion of the brain but exhibiting moderate to high mitotic rates with nuclear atypia and areas of necrosis. E and F. Benign adenoma with little atypia or necrosis and fewer mitotic figures.

Figure 2. Hyperpigmentation in $Rb^{+/-}$ and $p27^{-/-}$ mice. A) An 11 month old $Rb^{+/-}$ mouse 2 weeks after initial manifestation of darkened vibrissae. Note onset of dorsal patch. This animal is beginning to show signs of emaciation. B) 9 month old $p27^{-/-}$ mouse with approximately 70% dorsal hyperpigmentation. 2 weeks earlier the periorbital patches were isolated islands of pigmentation. The animal is still in good condition. C) Post mortem photograph of an emaciated $Rb^{+/-}$ mouse showing classical dark vibrissae with dark suborbital and dorsal patches. D) Close up of another post mortem photograph of a 12 month old $Rb^{+/-}$ mouse with bilateral supraorbital patches and darkened vibrissae.

Figure 3. Adrenal histology at 20 x, 200x and 600x magnification showing representative areas of the medulla (*m*) and the cortical zona glomerulosa (*zg*) and zona fasciculata (*zf*). A-C; Example of a normal adrenal from an $Rb^{+/-}$ mouse without hyperpigmentation but with a small pituitary adenoma. D-F; Examples from a $Rb^{+/-}$ mouse with hyperpigmentation associated with an aggressive adenoma greater than 4 mm. Note few cytoplasmic vesicles in the vast majority of the *zf* but the normal appearance of the adrenal medulla and cortical *zg*. G-I; Examples from a $p27^{-/-}$ mouse with a pituitary adenoma and hyperpigmentation. Note medullary hyperplasia and a mixture of cells in the *zf* both with and without cytoplasmic vesicles and some cells that contain cytoplasmic areas with and without vesicles.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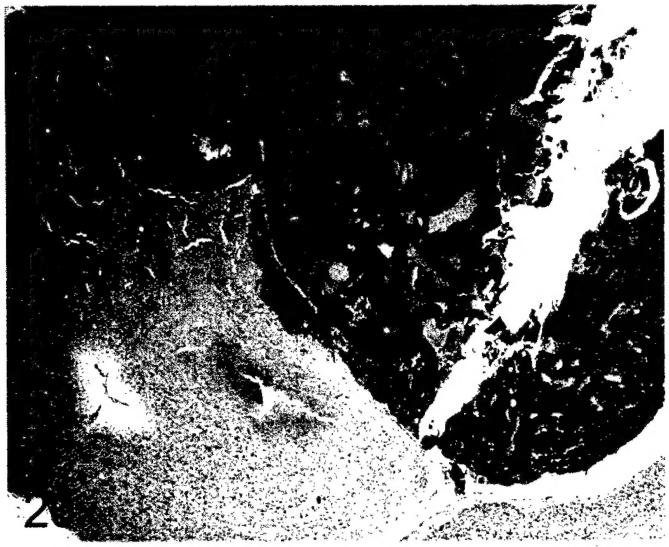

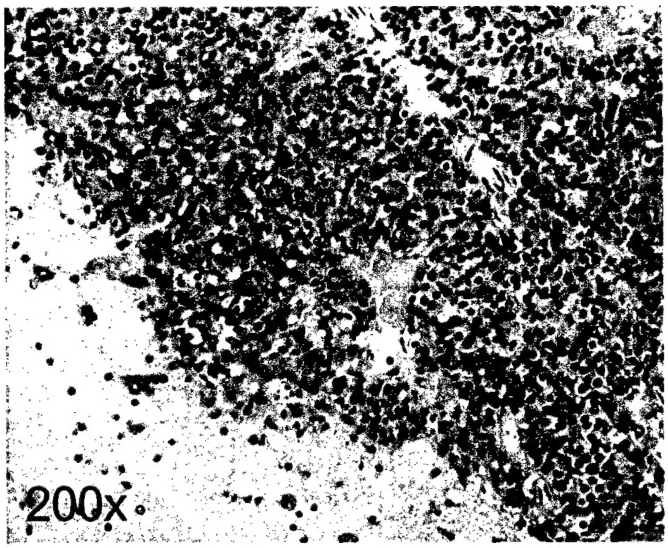
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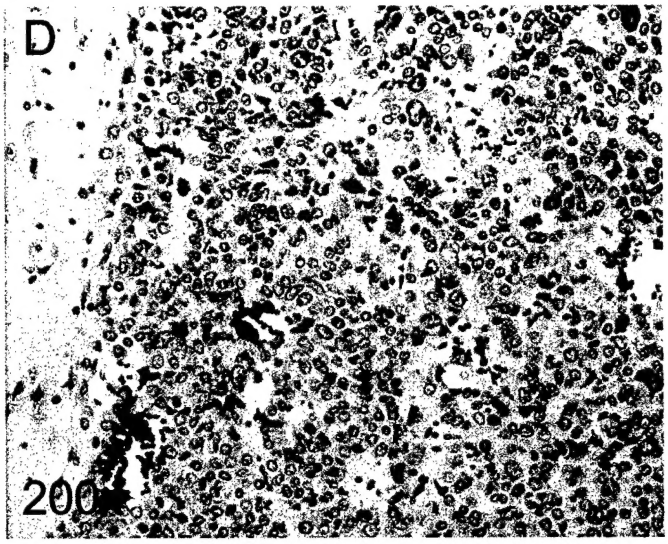
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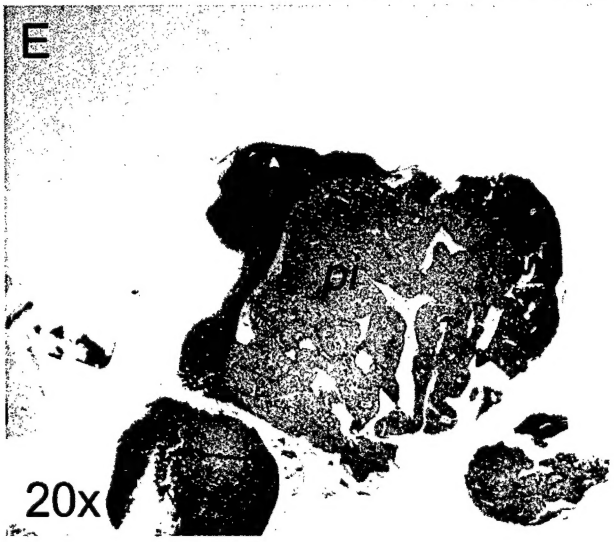
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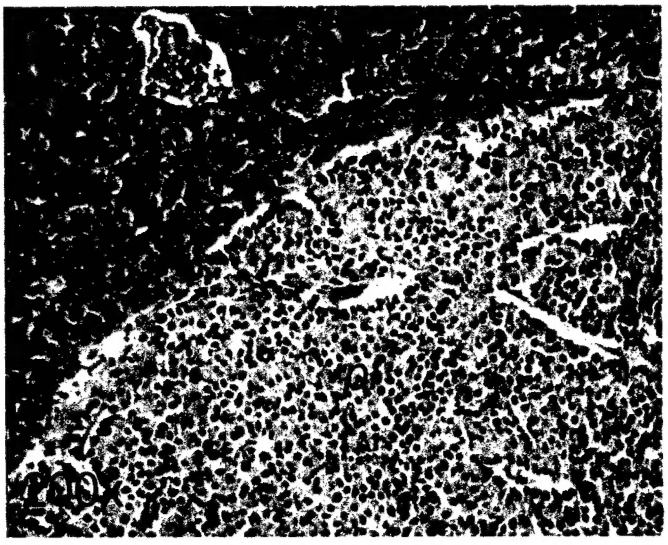

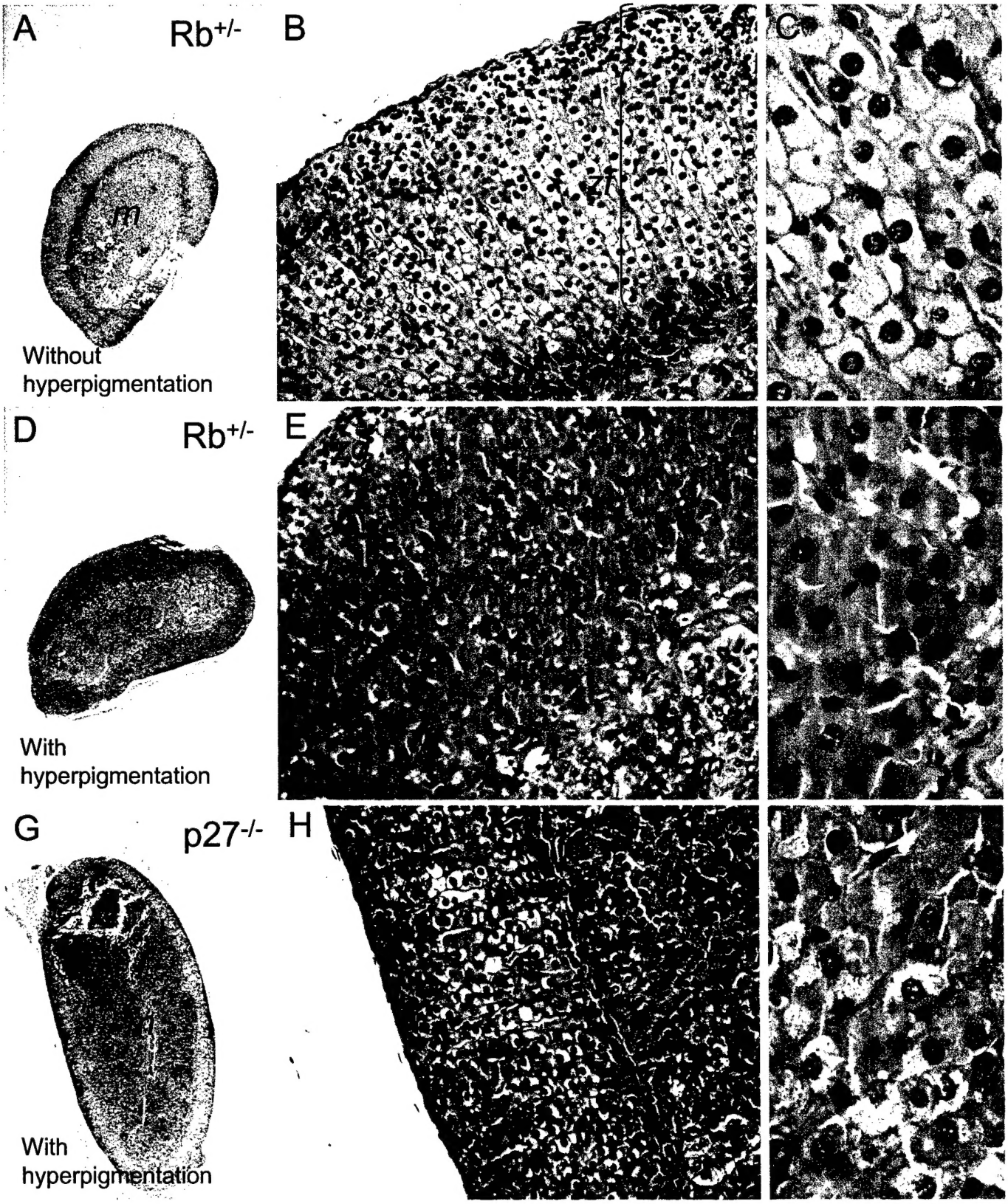
A
Rb+/-
B
C
Without hyperpigmentation
D
Rb+/-
E
With hyperpigmentation
G
p27-/-
H
With hyperpigmentation
20x
200x
600x